

Direct selection of antibodies that coordinate metals from semisynthetic combinatorial libraries

(metalloproteins/catalytic antibodies/surface expression/magnetite)

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ABSTRACT An iterative strategy for the selection of catalytic metalloantibodies is described. The first stage of this strategy is validated by the selection of semisynthetic antibodies that coordinate a variety of different metal ions and the metal oxide magnetite. These results have implications not only for the development of catalytic metalloantibodies but also for the development of reagents for magnetic resonance imaging, delivery of radioisotopes, and purification of recombinant proteins.

Metals are important cofactors in enzyme-catalyzed reactions such as phosphoryl and acyl group transfers, aldol condensations, and redox reactions. Indeed, about one-third of nature's enzymes utilize metals in structural or catalytic roles (1). In the realm of organic synthesis, metal-catalyzed reactions are among the only reactions that compete with enzymes in efficiency and ability to control the stereochemical outcome of organic transformations (2, 3). Thus, the incorporation of metal-binding sites into proteins is a step toward construction of metalloenzymes. To date, however, most metal-binding sites have been incorporated into proteins to impart stability (4), regulate activity (5), aid in purification (6), or mediate their assembly (7). The *de novo* design of catalytically active metalloproteins has yet to be accomplished as not only metal ligation but also transition state stabilization of the reaction must be considered and will likely remain out of reach for all but the simplest reactions.

Our interest lies in the *de novo* creation of catalytic antibodies. To further this goal, we have begun to develop approaches that allow experimenter-controlled evolution of protein architecture (8–10). These random *in vitro* methods are particularly applicable to the selection of antibodies that directly coordinate metal cofactors in their combining sites and differ from our previous approach in which a metal coordination site was designed into an antibody light chain of known structure (11, 12). Here we report the selection of antibodies from semisynthetic combinatorial antibody libraries that ligate metals and metal oxides in their combining sites.

MATERIALS AND METHODS

Reagents, Strains, and Vectors. Metal salts, iron(II, III) oxide, iminodiacetic acid (IDA), and 1,4-butanediol diglycidyl ether were obtained from Aldrich. Chelating Sepharose was obtained from Pharmacia. Oligonucleotides were from Operon Technologies (Alameda, CA). *Escherichia coli*, phage, and the phagemid vector pComb3 are as described (8, 9).

Library Construction. Heavy-chain complementarity-determining region 3 (HCDR3) libraries were prepared as described (10). The following primers were used in place of previously described primer 7ECDR3: HCDR10, 5'-GTG TAT TAT TGT GCG AGA (NNS)₁₀ TGG GGC CAA GGG

ACC ACG-3' and 5'-GTG TAT TAT TGT GCG AGA (NNS), TGG GGC CAA GGG ACC ACG ACG-3'. PCR was performed with 10–20 independent reaction pools as before, with the same template DNA, pC3TT7E. The resulting libraries were designated 7-10 and 7-5, respectively. In an analogous fashion, additional libraries were prepared by using the template DNA of clone 1 described in ref. 8. In these cases, primer BFR3U, 5'-TCT CGC ACA GTA ATA CAC GGC CGT-3', was used in place of B7EFR3. In place of 7ECDR3, the following primers were used: HCDR16, 5'-GCC GTG TAT TAC TGT GCG AGA GGT (NNK)₁₃ GAC NNK TGG GGC CAA GGG ACC ACG GTC-3'; HCDR10, 5'-GCC GTG TAT TAC TGT GCG AGA GGT (NNK), GAC NNK TGG GGC CAA GGG ACC ACG GTC-3'; HCDR15, 5'-CGG GTG TAT TAC TGT GCG AGA (NNK)₃ GAC NNK TGG GGC CAA GGG ACC ACG GTC-3', which resulted in the production of libraries E, F, and G, respectively. In addition, primer R3B, 5'-TTG ATA TTC ACA AAC GAA TGG-3', was used in place of CG1Z. All subsequent manipulations were as described (10). After phage precipitation, the phage pellet was resuspended in 25 mM Mops/150 mM NaCl/1% bovine serum albumin (BSA), pH 7.4.

Selection of Magnetite-Binding Fab Fragments. Phage libraries prepared as described above were applied to 1 mg of magnetite in a microcentrifuge tube. The resulting suspension was shaken at 37°C for 2 hr. Magnetite was pelleted by centrifugation or magnetic force. Magnetite was washed 5 times with Tris-buffered saline (TBS)/Tween in the first three rounds of selection and 10 times in the last three rounds of selection. Bound phage were eluted with the standard acidic elution buffer. Subsequent steps are as described (8, 9).

Selection of Metal-Binding Fabs. Selection for metal binding using Sepharose beads proceeded in a fashion analogous to biopanning, where the target antigen is immobilized on a microtiter plate. Chelating Sepharose (10 mg dry weight) was loaded by suspension in a 10 mM solution of CuSO₄, ZnCl₂, CeCl₃, FeCl₃, or Pb(NO₃)₂ in water. Excess metal was removed by washing the resin twice with TBS. Phage libraries in 25 mM Mops/150 mM NaCl/1% BSA were applied to the loaded Sepharose and incubated at 37°C with shaking for 2 hr. For the initial selection, beads were washed once with Mops-buffered saline. Subsequently, beads were washed 5 times for rounds 2 and 3 and 10 times for each additional round of selection. Bound phage were eluted once with elution buffer supplemented with 1 mM metal and once with 50 mM EDTA to remove excess metal from the resin. The 10-fold increase in background binding, which is presumably due to the increased surface area of the beads, necessitated additional rounds of selection, generally five or six as compared to three or four rounds of selection to protein antigens and small organic haptens.

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Abbreviations: BSA, bovine serum albumin; IDA, iminodiacetic acid; CDR, complementarity-determining region; HCDR3, heavy-chain CDR3.

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Preparation of BSA-IDA Conjugate. A 10-ml solution of 0.1 M carbonate buffer (pH 9) was prepared containing 10 mg of BSA per ml. 1,4-Butanediol diglycidyl ether (0.9 mmol) was added to this solution and allowed to react for 4 hr at 37°C. IDA (1.8 mmol) neutralized with NaOH and dissolved in 3 ml of 0.1 M bicarbonate (pH 9) was then added to the protein-containing solution and allowed to react overnight at 37°C. The conjugate was purified by passage over a Pharmacia PD-10 Sephadex column.

ELISA with BSA-IDA Conjugate. BSA-IDA (1 µg) diluted in TBS was applied to each well of a 96-well ELISA plate. The plate was allowed to incubate overnight at 4°C or for 1 hr at 37°C. The coating solution was removed and 50 µl of 1% BSA in TBS was applied. After blocking for 30 min at 37°C and removal of the BSA solution, 40 µl of 1 mM EDTA was applied to all wells to chelate preexisting metal ions. After removal of this solution, 40 µl of a 1 mM solution of a metal salt ($MgCl_2$, $NiCl_2$, $CuCl_2$, etc.) in TBS was added. After metal loading, the plate was washed 5 times with purified water and dried, and primary Fab was added. After 2 hr at 37°C, the plate was washed 10 times with phosphate-buffered saline (PBS)/Tween (0.05%). Goat anti-human IgG F(ab')₂ coupled to alkaline phosphatase (40 µl of a 1-µg/ml solution) in 1% BSA/TBS was then applied to each well. After 30 min at 37°C, the plate was washed 10 times with PBS/Tween (0.05%), and substrate [*p*-nitrophenyl phosphate (0.1% in 10% diethanolamine)/0.01% $MgCl_2$, pH 9.8] was added. Absorbance was measured at 405 nm.

Estimation of the Formation Constant for Fab Cu-2 and Ni-Loaded BSA-IDA. The formation constant was determined by a competition ELISA experiment. A stock solution of 20 µM BSA-IDA was saturated with $NiCl_2$ in TBS. This metal-loaded BSA-IDA solution was serially diluted to 0.2 nM. One hundred microliters of each BSA-IDA-Ni solution was preincubated with 100 µl of Fab Cu-2 at 2 µg/ml to give final BSA-IDA-Ni concentrations of 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 5×10^{-6} , and 10^{-5} M. Forty microliters of Fab/competitor was applied in quadruplicate to ELISA wells, which had been coated with BSA-IDA-Ni (as described). Normal ELISA protocols were then followed. The formation constant was defined as the reciprocal of the concentration of BSA-IDA-metal that reduced A_{405} to half-maximal.

RESULTS

Library Construction. The human tetanus toxoid-binding Fab 7E(10) and 1(8) were used as templates for CDR-directed mutagenesis. Clone 1 was chosen because it is easily produced in *E. coli*. PCR-mediated gene construction and subsequent steps to prepare antibody libraries were performed as described. Each unique library consisted of at least 10^8 independent clones derived from five transformations. Sequence analysis of DNA from unselected libraries confirmed the targeted mutagenesis and the desired distribution of bases. Selection for binding to Cu(II) and magnetite utilized all six libraries in a competitive fashion such that in the first round of selection libraries were kept separate until the ligation step, when binding phage were combined for amplification and subsequent selections. Sequence analysis of the resulting binding phage revealed that all were derived from libraries E and F. Library 7-16 was the original source of semisynthetic fluorescein-binding Fabs. In subsequent selections, only libraries E and F were examined. These libraries retain the aspartate in the penultimate position in the HCDR3 and their success supports the structural significance of this residue within HCDR3 (10).

Selection for Binding to a Metal Oxide and Sequence Analysis. After six rounds of selection, the enrichment in binding is indicated by the increase in the number of eluted phage as compared to the first round was 100. Electron microscopic studies showed that selected but not control phage bind

LERRTVKHHVNLE

lamB loop mutant

SRRSRHHPRMWNGLDV

Antibody HCDR3

GRFKRVDRWVVIFDF
GVARSKKMRGLWRLDV
GLAVRSKGRGRFFLFDV

LEGRIRRSLKLRL

lamB loop mutant

FIG. 1. Comparison of amino acid sequences of the HCDR3 regions selected for binding to magnetite with *lamB* mutants, which were selected in a similar manner (13). Boxed regions highlight the similarities between the clones. Underlined region of the third clone highlights an unusual basic region.

magnetite (data not shown). Sequencing of 8 clones revealed four unique sequences (Fig. 1). The RRSRHH- and RSKRGR-containing sequences were found three times each (identical at the nucleotide level). All clones were derived from libraries of HCDR3 length 16. Furthermore, all are derived from library E, where the first and penultimate amino acid residues are fixed as glycine and aspartate, respectively. In clone 1, the serine at the first position is likely an artifact of the synthesis and assembly and is the result of a single base change (GGT → AGT). This base change is seen in other clones selected for metal ion binding. The clones show a number of consensus features. The N-terminal portions of all the loops are rich in basic amino acids, whereas the C terminus is rich in hydrophobic residues. Three of four clones bear arginine at position 9 and valine at position 16. Two clones have an RSK triplet and one has an RSR triplet.

Characterization of the Specificity and Affinity of Metal-Binding Clones. Three Cu(II)-selected antibodies were purified. BSA-IDA-metal ELISAs were performed to analyze their binding properties. All three bound Cu(II)-loaded BSA-IDA significantly over background. Interestingly, two of these demonstrated a marked preference for Ni(II) over all the other metals examined (Fig. 2). Each of the three Fabs had unique specificity characteristics, as one might expect from a random selection procedure. These results highlight the diversity that still exists in the metal-binding sublibrary. The binding specificity of the Pb(II)- and Ce(III)-selected Fabs was also confirmed by ELISA. Analysis of the sequences of the selected antibodies confirms the success of the selection strategy (Fig. 3). This is particularly obvious with

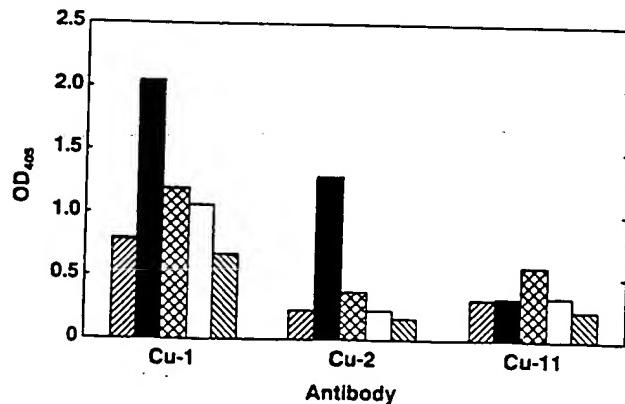


FIG. 2. Comparison of binding specificities of three purified clones that were selected for Cu(II) binding. Specificity was examined by ELISA. Antigens were (from left to right) Mg(II) BSA-IDA, Ni(II) BSA-IDA, Cu(II) BSA-IDA, Zn(II) BSA-IDA, and BSA-IDA (no metal).

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Cu(II)	Zn(II)	Pb(II)
GRVHHHSLDV	SHTHALPLDF	GNLRRKTSI
SWKHHAHWDV	GRVHHHSLDV	GESDSKREDG
GSDWDRGCDG	GQSSGGDTDD	GGPSLAVGDW
GHHMYGGWDH	GQWTPRGDDF	GPLQHTYPDY
GHWGRHSLDT	GRCCPSSCDE	GWKVTAEDSTEGLFDL
GHILHHQLDL	GPAKHRHRHVQMHDS	GTRVWRVCQWNHEEDG
SSQRMLMGDN		GEWWCSFAMCPARWDF
SHHGHHYLNH		GDTIFGVTMGYYAMDV
GKLMMMSWC RDTEGCDH		
GDTHRGHLRHHLPHDW		
GWGLWMKP FVWRRAWDM		
Ce(III)		Fe(III)
GQVMZELGDA		SASMRSAIGLWRTMDY
GLTEZQLQDG		GDREIFHMZWPLRVVDV
GYSYSVSPDA		SZNPQQVCGVRCGZDK
GRLGLVMTDE		GNRLSSSGHLLKQGZDG
STWPGRQRLGZALSDS		GGSDWZIGACCREDL
GYELSWGVDZQEWWDI		GMVSMMGQS RPTQCDC
GPVRGLDZSKGVRYDN		GVIKWIRRWRVRTARDV
GLSZHI VSETQSSGDL		GWFWRLLPTPRAPS DV
GLESLKVLGVZLGGDL		
GNMILGGPGCWSSADI		
GCWNVZR L VVYHPPDG		
GFEVTCSWF GHWRD S		

FIG. 3. Amino acid sequences of HCDR3 regions selected for binding various metals. Z represents an amber stop codon, which, in the *supE* strain used for expression, is translated as Q.

the Cu(II)-chelating sequences. Based on the knowledge of characterized copper-containing protein structures, the expected ligands are histidine, methionine, and cysteine. A total of 106 positions within the 11 sequenced Cu(II)-selected CDRs were randomized with an NNK doping scheme. Histidine was selected at 26 positions, methionine at 6 positions, and cysteine at 3 positions. Note that an NNK mixture provides each of these residues at only 1 part in 32 in the unselected library. One clone contains an unpaired cysteine residue, which is a rare feature in antibody CDRs (14). By utilizing IDA immobilized with a 12-atom spacer, selection should not be limited to surface-exposed residues. Three clones contain a histidine at position 102, which is predicted not to be on the surface of the protein (15). The two histidine-rich clones demonstrated a preference for Ni(II)-loaded BSA-IDA, while only the tryptophan-rich clone showed selectivity for Cu(II). The formation constant of clone Cu-2 for Ni(II) BSA-IDA was estimated by competition analysis to be 10^7 M^{-1} . Previous studies indicate that the formation constant for a single histidine with Cu(II) IDA-polyethylene glycol is $\sim 10^3 \text{ M}^{-1}$ (16). Binding was dependent on metal and was abolished by addition of EDTA and competed with excess free metal. It was important to demonstrate that Fab selected for binding a solid support could also bind copper ions in solution. As such, a fluorescence quenching experiment was performed (11) on a representative, purified Fab. By Scatchard analysis (Fig. 4), Cu-8 binds copper with a dissociation constant of $10 \mu\text{M}$ and a valence of 1.03 binding sites per Fab. Sequences selected for binding to Zn(II), Pb(II), Ce(III), and Fe(III) are also shown in Fig. 3. Zn(II)-selected sequences show the histidine-rich character seen for Cu(II) and one sequence that is identical to that selected with Cu(II). Only one clone is rich in carboxylates. Selection for histidine, cysteine, aspartate, and glutamate is expected based on studies of natural proteins (17). The coordinating ligands from sequences selected for binding to Pb(II), Ce(III), and Fe(III) are less obvious. A number of features, however, can be noted. Pb(II) sequences are rich in aspartate and glutamate (10% of randomized residues). For

comparison, Ce(III)-, Fe(III)-, and Cu(II)-selected sequences contained these residues at 7%, 4%, and 5%, respectively. The oxygen containing ligands serine, threonine, and tyrosine constitute 18% of Pb(II) and Ce(III) sequences as compared to 11% and 9% for Fe(III) and Cu(II). Note that serine and threonine are overrepresented at the synthesis level and are present at 3 and 2 parts per 32. Fe(III) sequences were enriched to the 10% level for the sulfur-containing residues cysteine and methionine as might be anticipated (18). Coordination with main-chain carbonyl oxygens or amide nitrogens cannot be discounted and would not be discernible from sequence analysis.

DISCUSSION

Within the pool of 20 commonly occurring amino acids almost half have been observed to participate in metal ligation within proteins (aspartate, cysteine, glutamate, his-

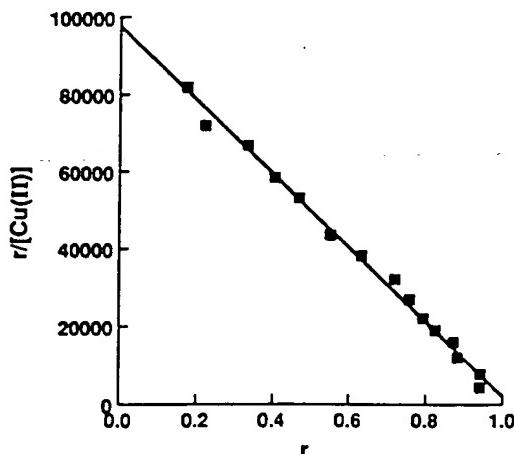


FIG. 4. Scatchard analysis of fluorescence quench data for Fab Cu-8 binding to copper in solution. The data can be fit with $r^2 = 0.994$ by the equation: $r/[Cu] = -94,548r + 97,073$.

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tidine, methionine, serine, threonine, tyrosine, and tryptophan). Main chain carbonyl oxygens and amide nitrogens can also be used to coordinate metals. Most protein engineering efforts utilize design strategies based on analogy (1). While such a strategy may be sufficient for the transfer of known metal-binding motifs into alternative proteins, it limits one's ability to explore and exploit novel reactivities for most of the periodic table. The ability to selectively sort proteins *in vitro* for metal ligation has the distinct advantage that it is not limited in scope by natural phylogenies. Furthermore, we demonstrate here that selective approaches generate a variety of ligating groups, which should provide a multiplicity of coordination numbers and geometries that alter the reactivity of the bound metal.

A general iterative strategy for selection of catalytic synthetic antibodies is shown in Fig. 5. The first step in this strategy involves selection of sequences within the antibody-combining site that coordinate metal. The preferred regions in which mutagenesis is performed are the CDRs (10). In this

initial report, we have used the HCDR3. Selection is most easily achieved by immobilization of the target. In our case, the immobilization of a metal ion utilized a support derivatized with a chelating agent. Such IDA supports are suitable for immobilization of many metals. Furthermore, this chelating agent occupies a limited number of coordination sites on the metal and provides the metal with a number of vacant sites (H_2O), which are used to select coordinating antibodies from the library. As an alternative to an inert support, transition-state analogs could be synthesized that immobilize the metal for selection.

The second step involves diversification of the library either by chain shuffling or mutagenesis of another CDR. From this pool of antibodies, which coordinate metal, a second selection is used to optimize binding to a hapten, which may also provide a site to coordinate metal, but formal coordination to the substrate is not necessary so long as the metal participates in the chemistry. For example, a Zn atom coordinated to one CDR may be used to deliver a hydroxide

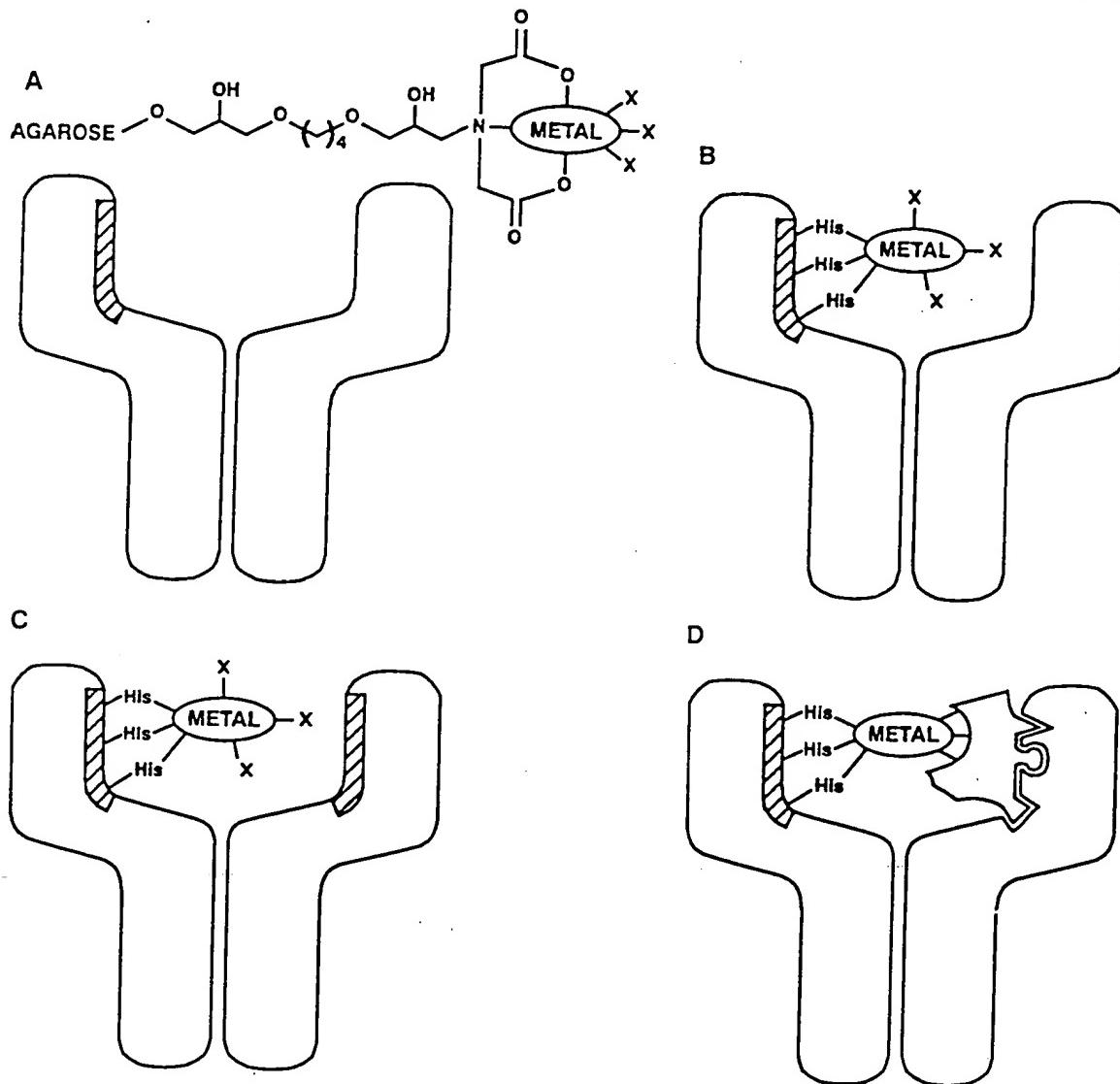


FIG. 5. An iterative strategy for selection of catalytic synthetic antibodies. (A) Antibody-binding site formed by dimerization of heavy and light chains. Hatched region indicates an antibody CDR that has been targeted for mutagenesis. The resulting library of antibodies is then selected to bind the metal-IDA complex shown at the top of A, where X designates a vacant (H_2O) coordination site. The antibodies that result are shown in B, where the vacant X sites of the complex shown in A are substituted for histidine ligands in this example. Coordination sites formerly occupied by IDA are now vacant (X). (C) Diversification of the metal-binding antibodies by mutagenesis of another CDR or chain shuffling. The resulting sublibrary is then selected for binding to an appropriately designed hapten to produce catalytic metalloantibodies shown in D to bind both metal and hapten.

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ion to the carbonyl carbon of a peptide bound to another region of the antibody molecule. Light-chain CDR3 may be the most desirable region to install metal binding as the extended HCDR3 could be used to provide the hapten-binding pocket. It is interesting to note that most known copper-containing proteins coordinate copper within a loop of a Greek key folded domain (19). Antibodies exhibit this same fold and a naturally occurring copper-binding antibody derived from a myeloma patient has been described (20). A Hg(II)-binding antibody has also recently been described (21).

Many of the metals we have introduced into the antibody-combining site could be useful in catalysis. Cu(II) has been used to catalyze nonoxidative hydrolysis reactions as well as oxidative reactions (22, 23). Fe(II) is particularly interesting for the oxidative cleavage of proteins and could be used as a cofactor to produce antibodies that cleave proteins at defined sequences (24). Pb(II) and Ni(III) have been used to cleave RNA and DNA, respectively (25, 26). One of the most interesting results concerns the lanthanide cerium. Ce(III) hydroxide clusters have recently been demonstrated to catalyze the hydrolysis of cAMP with a 10¹¹-fold rate enhancement (27). This makes Ce a particularly interesting metal to incorporate into antibodies in an effort to produce artificial restriction enzymes. Furthermore, Ce serves as an example of a catalytically interesting metal for which there is no known naturally occurring protein from which to design a metal-binding site.

The selection of antibodies that bind the iron oxide magnetite demonstrates that proteins can be selected to bind surface features of a mineral. Interestingly, in this case there exists a report in the literature of the selection of *E. coli* that adhere to magnetite, which can be used for comparison (13). In this experiment, random nonapeptide sequences were inserted into an exposed portion of the *E. coli* bacteria lamB protein and selected for binding to magnetite. Fig. 1 shows the striking similarity in sequence of selected antibody CDR and lamB variants selected in a similar fashion. Two clones bear an RSK sequence, which was identified in the previous report to discriminate between different iron oxides. A third clone contains an RRSRHH sequence as compared to the lamB-selected clone bearing RRTVKHH. The clones share a number of other consensus features. Our selection process was evidently stringent, as only four unique sequences were isolated from a pool of >10⁸. Since naturally occurring bacteria that accumulate magnetite have been identified, it would be interesting to know whether the artificially selected motifs are involved in natural proteins, which have been selected during evolution (28). At any rate, these studies may further indicate that for certain coordination complexes there are limited opportunities within the context of the folded protein. Magnetic antibodies might be more directly applicable than the recently synthesized magnetoferitin (29) in magnetic imaging and separation schemes. Other practical applications for the metal ion selection procedure include optimizing proteins for delivery of radioisotopes and engineering recombinant proteins for rapid purification on immobilized metal supports.

We have demonstrated the efficiency with which the first stage of an iterative approach to catalytic metalloantibodies can be completed. A benefit of such an evolutionary approach, as opposed to a designed template approach, is that

sublibraries can be created whose members display a diversity of coordinating residues in a range of potentially useful contexts. The advantage of creating such sublibraries stems from the power of developing selective approaches that allow rare but interesting members to be further selected based on criteria that can be entirely different than those used to create the original library. We believe that iterative, evolutionary approaches such as that presented here will prove useful for the creation of new catalytic antibodies.

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